

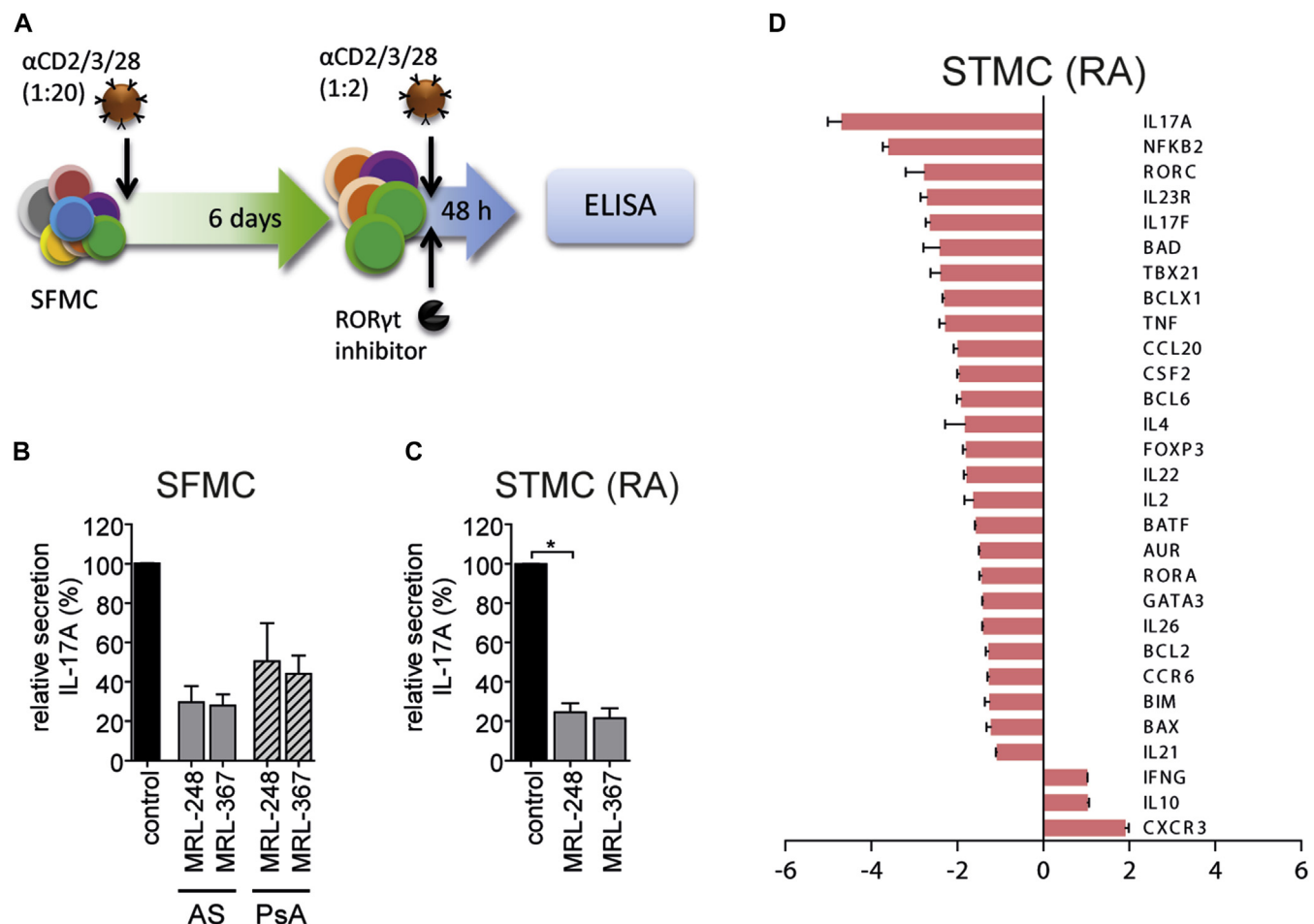


FIG 2. RORγt inhibition suppresses IL-17A secretion in synovial-derived cells. **A**, Mononuclear cells from the synovial fluid (SFMC) of patients with AS and PsA were cultured in T_H17-promoting conditions for 6 days and subsequently restimulated for 48 hours with anti-CD2/anti-CD3/anti-CD28 beads in the presence of RORγt inhibitors. **B**, Secreted IL-17A (ELISA) measured from patients with AS (n = 3) or PsA (n = 3). Data are mean + SEM and relative to control conditions. **C**, Mononuclear cells were obtained from a 14-day explant culture of synovial tissue (STMC) derived from patients with RA and subsequently cultured in the presence of anti-CD2/anti-CD3/anti-CD28 beads and RORγt inhibitors (see cartoon Fig E10). IL-17A secretion was measured after 48 hours. Data are mean + SEM from 4 patients with RA; *P < .05 by Friedman test. **D**, RT-PCR was performed on mRNA isolated from synovial tissue cells of patients with RA after 48-hour stimulation in the presence of MRL-367. Three technical replicates of n = 1, mean + SD, are shown.

We here describe 2 novel indazole small-molecule RORγt inhibitors, MRL-248 and MRL-367 (Fig 1, A), which inhibit steroid receptor coactivator 1 peptide binding to RORγt with IC₅₀ potency of 1 to 2 nM in a fluorescence resonance energy transfer (FRET)-based assay (see Table E1 in this article's Online Repository at www.jacionline.org). RORγ/Gal4 cell-based reporter assays showed inhibition of RORγt by MRL-248 and MRL-367 with IC₅₀s of 118 nM and 41 nM, respectively (Table E1). In contrast, the RORγt inhibitors digoxin, TMP778, and SR1001 have shown IC₅₀s in the micromolar range.⁷ MRL-248 and MRL-367 showed no appreciable activity against a panel of related nuclear hormone receptors (see Table E2 in this article's Online Repository at www.jacionline.org).

Next, we tested the suppression of the T_H17 function of human total CD4⁺ T cells from healthy donors by MRL-248 and MRL-367. Both RORγt inhibitors reduced IL-17A⁺ T-cell numbers in flow cytometry-based assays in a comparable and dose-dependent manner (see Fig E1 in this article's Online

Repository at www.jacionline.org). Both T_H17 skewing from naive CD4 T cells (see Fig E2, A, in this article's Online Repository at www.jacionline.org) and polarization of memory CD4 T cells (Fig E2, B) was inhibited by MRL-248 and MRL-367, without global effects on cellular proliferation (Fig E2, C). Secretion of IL-17A by established CCR6⁺CD161⁺ T_H17 cells was also reduced by both inhibitors (see Fig E3 in this article's Online Repository at www.jacionline.org).

We next investigated the ability of RORγt inhibitors to suppress IL-17A secretion by T_H17 cells derived from patients with inflammatory arthritis, patients with IBD, and healthy controls. We cultured total PBMCs (as more representative of the *in vivo* situation) for 6 days with suboptimal T-cell stimulation and IL-2, resulting in T_H17 expansion while preserving T_H1 frequencies (see Fig E4 in this article's Online Repository at www.jacionline.org and data not shown). RORγt inhibitors suppressed the secretion of IL-17A by 70% in cells derived

from patients with AS (Fig 1, B). Healthy control PBMC cultures produced less IL-17A, but were also profoundly inhibited (see Fig E5 in this article's Online Repository at www.jacionline.org). The number of IL-17A⁺ cells within the CD4⁺ T-cell compartment was reduced by up to 50% by both MRL-248 and MRL-367 in all 4 inflammatory diseases: AS, PsA, RA, and IBD (Fig 1, C and D; see Fig E6 in this article's Online Repository at www.jacionline.org). A significant fraction of T_H17 cells coexpressed other cytokines, including IFN- γ , IL-22, and GM-CSF. For AS, PsA, RA, and IBD, both ROR γ t inhibitors suppressed polyfunctional (and potentially pathogenic) IL-17A⁺ cells coproducing IFN- γ , IL-17F, IL-22, GM-CSF, or TNF, but not cells expressing these cytokines independently of IL-17A (see Fig E7 in this article's Online Repository at www.jacionline.org), although conversion of double to single producers cannot be excluded. Although most of the IL-17A-producing cells were CD4⁺ T cells, ROR γ t inhibition also suppressed the IL-17A⁺ population of AS, PsA, and IBD CD8⁺ T cells (see Fig E8 in this article's Online Repository at www.jacionline.org).

We next determined the effects of ROR γ t inhibition on the transcriptional profile of T_H17 cells from patients with AS and RA. MRL-367 and MRL-248 significantly reduced the transcription levels of T_H17-associated genes, including *IL17A*, *CCL20*, *CCR6*, *IL17F*, *IL23R*, and *IL22* (see Fig E9 in this article's Online Repository at www.jacionline.org), concurring with previous reports.^{8,9} Interestingly, MRL-248 and MRL-367 markedly reduced *IL26* transcription in T_H17 from both patients with AS and RA. This reduction was not reported for patients with psoriasis treated with TMP778.⁹ The proinflammatory cytokine IL-26 has previously been associated with RA and IBD, but to our knowledge not with AS. We propose that IL-26 may constitute a pathogenic T_H17-associated cytokine in AS and merits further study.

Transcription of other T_H subset-associated genes (such as *IFN- γ* and *IL4*) or transcription factors (*TBX21*, *GATA3*, *FOXP3*, and *BCL6*) was not reduced by ROR γ t inhibitors, confirming a specific effect on type 17 cells. *IL10* transcription was modestly enhanced, supporting the inverse correlation between ROR γ t and Foxp3, although this finding requires confirmation due to the small study size. Overall, these transcriptional data confirm the cytokine inhibitory effects observed by ELISA and intracellular cytokine staining, and also identify new candidates meriting further study.

The effects of ROR γ t inhibition were tested on CD4⁺ T cells derived from the inflamed tissue of patients with inflammatory arthritis. Mononuclear cells were isolated from the synovial fluid of 3 patients with AS and 3 patients with PsA and cultured for 6 days in T_H17-promoting conditions (Fig 2, A). Restimulation of these cells in the presence of ROR γ t inhibitors suppressed IL-17A secretion by 75% (Fig 2, B). Last, we analyzed the efficacy of the inhibitors on T cells obtained after 2-week culture of synovial tissue explants from patients with RA (see Fig E10 in this article's Online Repository at www.jacionline.org). Once again, restimulation of inflamed tissue-derived cells in the presence of ROR γ t inhibitors resulted in a reduction in IL-17A secretion (Fig 2, C). Transcriptional analysis of T_H17 cells derived from the synovial tissue of patients with RA confirmed downregulation of T_H17 signature genes by ROR γ t inhibition (Fig 2, D; see Fig E11 in this article's Online Repository at www.jacionline.org).

The novel ROR γ t inhibitors described here consistently and specifically inhibit the production of IL-17A and coproduced pathogenic cytokines by T_H17 cells *ex vivo* from patients with active AS, PsA, RA, and IBD, while sparing other T-cell immune pathways. Our findings provide a strong rationale for therapeutic trials of ROR γ t inhibition in inflammatory arthritis and IBD.

Merck Research Laboratories provided MRL-248 and MRL-367.

Jelle de Wit, PhD^{a,*}
M. Hussein Al-Mossawi, MD^{a,*}
Michael H. Hühn, PhD^b
Carolina V. Arancibia-Carcamo, PhD^b
Karen Doig, RGN^a
Benjamin Kendrick, MD, PhD^a
Roger Gundle, MD, PhD^a
Peter Taylor, MD, PhD^c
Terri Mcclanahan, PhD^d
Erin Murphy, BSc^d
Hongjun Zhang, PhD^e
Ken Barr, PhD^e
J. Richard Miller, PhD^e
Xiao Hu, PhD^e
Thomas D. Aicher, PhD^f
Rodney W. Morgan, MD^f
Gary D. Glick, PhD^g
Dennis Zaller, PhD^e
Craig Correll, PhD^e
Fiona Powrie, PhD^{b,h}
Paul Bowness, MD, PhD^{a,c}

From ^aNuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom; ^bthe Translational Gastroenterology Unit, Nuffield Department of Clinical Medicine, Experimental Medicine Division, John Radcliffe Hospital, University of Oxford, Oxford, United Kingdom; ^cNational Institute for Health Research, Musculoskeletal Biomedical Research Unit, Nuffield Orthopaedic Centre, University of Oxford, Oxford, United Kingdom; ^dMerck Research Laboratories, Palo Alto, Calif; ^eMerck Research Laboratories, Boston, Mass; ^fLycera Corp, Ann Arbor, Mich; ^gthe Department of Chemistry, University of Michigan, Ann Arbor, Mich; and ^hKennedy Institute of Rheumatology, Nuffield Department of Orthopaedics, Rheumatology and Musculoskeletal Sciences, University of Oxford, Oxford, United Kingdom. E-mail: dewitjelle@gmail.com.

*These authors contributed equally to this work.

Disclosure of potential conflict of interest: M. H. Al-Mossawi has received research and travel support from Wellcome Trust, funds from Merck Research Laboratories, and research support from Oxford University Clinical Academic School and Oxfordshire Health Services Research Committee. K. Doig received funds from the Comprehensive Local Research Network. J. de Wit has received funds from the National Institute for Health Research (NIHR). J. de Wit, M. H. Hühn, C. V. Arancibia-Carcamo, P. Bowness, T. Mcclanahan, E. Murphy, H. Zhang, J. R. Miller, C. Correll, and D. Zaller have received research support from Merck Research Laboratories, Oxford University. P. Taylor has served as a consultant to Merck. T. Mcclanahan is employed by Merck. J. R. Miller is employed by and has stock/stock options in Merck. X. Hu is employed by Lycera; Lycera has an upfront collaboration with payment from Merck. T. D. Aicher is employed by, has vested stock options in, and has received travel support, fees for participation in review activities, payment for writing/reviewing the manuscript, and provision of writing assistance from Lycera; Lycera and Merck have a business partnership on ROR γ and several related patents. R. W. Morgan is employed by Lycera Corp. F. Powrie has received research support from Merck and has received consultancy fees from Effimune. P. Bowness is funded by the Oxford NIHR Biomedical Research Centre. P. Taylor and P. Bowness thank Arthritis Research UK for their funding of Arthritis Research UK Early Arthritis Treatment Centre at the University of Oxford and the NIHR for their funding of the NIHR Biomedical Research Centre in Musculoskeletal Disease at Oxford University Hospitals NHS Trust and the University of Oxford. This research has been conducted using samples from the Oxford IBD Cohort and the Oxford GI Illness Biobank. The rest of the authors declare that they have no relevant conflicts of interest.

REFERENCES

1. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol* 2009;27:485-517.

2. Lees CW, Barrett JC, Parkes M, Satsangi J. New IBD genetics: common pathways with other diseases. *Gut* 2011;60:1739-53.
3. Mease PJ, Genovese MC, Greenwald MW, Ritchlin CT, Beaulieu AD, Deodhar A, et al. Brodalumab, an anti-IL17RA monoclonal antibody, in psoriatic arthritis. *N Engl J Med* 2014;370:2295-306.
4. Genovese MC, Durez P, Richards HB, Supronik J, Dokoupilova E, Mazurov V, et al. Efficacy and safety of secukinumab in patients with rheumatoid arthritis: a phase II, dose-finding, double-blind, randomised, placebo controlled study. *Ann Rheum Dis* 2013;72:863-9.
5. Hueber W, Sands BE, Lewitzky S, Vandemeulebroecke M, Reinisch W, Higgins PD, et al. Secukinumab, a human anti-IL-17A monoclonal antibody, for moderate to severe Crohn's disease: unexpected results of a randomised, double-blind placebo-controlled trial. *Gut* 2012;61:1693-700.
6. Ivanov II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, et al. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. *Cell* 2006;126:1121-33.
7. Huh JR, Leung MW, Huang P, Ryan DA, Krout MR, Malapaka RR, et al. Digoxin and its derivatives suppress TH17 cell differentiation by antagonizing RORgammat activity. *Nature* 2011;472:486-90.
8. Xiao S, Yosef N, Yang J, Wang Y, Zhou L, Zhu C, et al. Small-molecule RORgammat antagonists inhibit T helper 17 cell transcriptional network by divergent mechanisms. *Immunity* 2014;40:477-89.
9. Skepner J, Ramesh R, Trocha M, Schmidt D, Baloglu E, Lobera M, et al. Pharmacologic inhibition of RORgammat regulates Th17 signature gene expression and suppresses cutaneous inflammation in vivo. *J Immunol* 2014;192:2564-75.

<http://dx.doi.org/10.1016/j.jaci.2015.09.048>

Repository Methods

Recombinant LBD of human and mouse ROR- γ

ROR γ ligand binding domains from human (Genbank accession number NP_005051 aa259-518) and mouse (NP_035411 aa236-516) were amplified by PCR and cloned into pET47b to include the sequence MAHHHHHHHHENLYFQGTPE N-terminal to the first residue of the ROR γ coding sequence. Proteins were expressed in BL21(DE3) *E. coli* grown in LB media. Expression was induced with 0.1 mM IPTG for 16 hours at 16°C. Cells were lysed in 50 mM Tris, 300 mM NaCl, 10 mM imidazole, 5% glycerol, 10 mM β -mercaptoethanol, 1% Triton X-100, 0.2 mM PMSF, pH 8.5 using a microfluidizer. After removal of cellular debris by centrifugation, lysates were loaded onto Ni-NDA resin and washed with 50 mM Tris, 300 mM NaCl, 20 mM imidazole, 5% glycerol, 10 mM β -mercaptoethanol at pH 8.5. The column was washed with the same buffer containing 60 mM imidazole, and protein was eluted with the above buffer containing 250 mM imidazole. The proteins were dialyzed into 50 mM Tris, 100 mM NaCl, 5% glycerol, 1 mM dithiothreitol, pH 8.5.

ROR γ -LBD SRC-1 cofactor peptide interaction assay

The potency of small molecule ROR γ ligands was assessed by monitoring their effect on the association of a LXXLL-motif containing steroid receptor coactivator (SRC1) peptide. MRL-248 was serially diluted using an Agilent Bravo liquid handler. Diluted compound or DMSO (25 nL) were transferred into a black Greiner 384 well plate and 15 μ L of 3.75 nM ROR γ -LBD in receptor buffer was added (50 mM Tris-HCl pH 7.0, 50 mM KCl, 1 mM EDTA, 0.1% delipidated bovine serum albumin, 1 mM dithiothreitol, 1.25 nM Anti-His Europium chelate (Perkin Elmer) and 3% (v/v) of lysate from ~24000 Sf9 cells). After 15 minutes incubation, 5 μ L of peptide (Biotin-SPSSHSLTERHKILHRLQEGSP; 250 nM) in detection buffer (50 mM Tris-HCL pH 7.0, 50 mM KCl, 1 mM EDTA, 0.1% delipidated bovine serum albumin, 1 mM dithiothreitol and 20 nM streptavidin-APC (Perkin Elmer)) or detection buffer alone were added and incubated overnight at 4°C. The plate was warmed to room

temperature and read using an Envision plate reader (Perkin Elmer). TR-FRET signal was defined as the ratio of the fluorescence emission at 615-665 nm following excitation at 337 nm. The percent activity of each dilution was determined as the ratio of background corrected signal to the background corrected signal of wells receiving only DMSO. IC₅₀ values were determined by fitting percent activity data to a four-parameter logistic dose response equation in Graph Pad Prism.

Chimeric ROR γ -GAL4 reporter assay

The coding sequence of ROR γ aa97-518 was cloned in frame with the DNA binding domain of the yeast GAL4 protein within the CMV-promoter-driven pCDNA3.1 vector. This vector, along with the GAL4 UAS-luciferase reporter vector pGL4.31 (Promega), were used to transfect HEK293T cells. Briefly, 1×10⁷ cells in 10 mL of DMEM high glucose media with 10% FBS were transfected with a mixture consisting of 10 μ g of each plasmid and 60 μ L of TransIT-293 (Mirus Bio) in 1.5 mL of Optimem (Invitrogen). Following transfection, cells were incubated overnight at 37°C and 5% CO₂. Test compounds were prepared as 10 mM stocks in DMSO and serially diluted 1:3 to provide an 8-concentration titration and 50 nL was transferred to a 384-well Greiner white tissue-culture treated plate. Cells were harvested and resuspended at 0.8×10⁶ cells per mL in DMEM high glucose media with 10% FBS. To each well of the plate 25 μ L of cell suspension was added and the cells incubated at 37°C and 5% CO₂. After 20-22 hours, the plates were brought to room temperature and 25 μ L of Steady-Glo luciferase reagent (Promega) was added to each well. The luminescent signal was measured on an Envision plate reader.

IC₅₀ assay on mouse splenocytes

Five spleens were obtained from C57BL6 mice and were macerated on a 70 micron nylon mesh filter. The cells were washed and resuspended in BD PharmLyse red blood cell lysing buffer and incubated for 3 minutes at 37°C. The cells were washed in RPMI + 10% FBS followed by passage through a 70 micron nylon mesh filter to remove connective tissue. Compound serial dilutions were prepared as

described above. Cells were added to each well and incubated for 30 minutes prior to addition of mouse IL-23 (10 ng/mL; R&D Systems), and then incubated for 18 hours at 37°C and 5% CO₂. Culture supernatants were collected and IL-17A levels were determined using a TR-FRET-based assay kit (CisBio).

Patients

Twenty-five milliliters heparinized venous blood was obtained from 35 patients with AS fulfilling the modified New York criteria, 16 patients with RA, 12 patients with PsA, 16 patients with IBD and 7 healthy individuals. Appropriate ethical permission (Centre of Research Ethical Campaign 06/Q1606/139 and Oxfordshire Research Ethics Committee B 07/Q1605/35, 11/YH/0020 and 09/H1204/30) and individual informed consent were obtained from patients and healthy controls participating in this study. Table E3 shows patient demographics and medication. Paired knee synovial fluid samples were obtained from three AS and three PsA patients.

Cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated using Histopaque (Sigma-Aldrich). CD4⁺ T cells were obtained by negative selection (Miltenyi or eBioscience). Were described, T cells were subsequently FACS sorted (>98% purity) for naïve (CD45RA⁺CD45RO⁻), memory (CD45RA⁻CD45RO⁺) or enriched Th17 (CCR6⁺CD161⁺). Cells were cultured in RPMI (Lonza) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-Glutamine (Sigma-Aldrich), with anti-CD2/anti-CD3/anti-CD28 beads (Miltenyi), in a 1:20 bead:cell ratio. Cells were cultured for six days in presence of Th17 polarizing cytokines IL-1β (20 ng/mL), IL-6 (20 ng/mL), IL-23 (20 ng/mL) and IL-2 (10 IU/mL; all Peprotech). Total PBMCs were cultured for 6 days in RPMI (Lonza) supplemented with 10% FBS, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-Glutamine (Sigma-Aldrich) in Th17-promoting conditions using suboptimal stimulation via anti-CD2/anti-CD3/anti-CD28 beads (1:20 ratio, Miltenyi

Biotec) and IL-2 (100 IU/mL, Peprotech). ROR γ t inhibitors MRL-248 and MRL-367 (1 μ M, Merck) were added to the culture when described.

Tissue

Tissue samples were obtained from surgical residual material from RA patients, which were gathered during a hip or knee arthroplasty or arthroscopy. Tissue was dissected into 1-3 mm³ cubes and cultured for 14-15 days in RPMI supplemented with 10% Human AB serum (Sigma-Aldrich), 100 U/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-Glutamine, in the presence of IL-2 (100 IU/mL) and IL-7 (10 ng/mL, Peprotech). Mononuclear cells migrated from the explant culture were harvested and 200,000 cells were stimulated for 48 hours with anti-CD2/anti-CD3/anti-CD28 beads and IL-2 (100 IU/mL). Cytokine secretion was measured by IL-17A ELISA (Ready-SET-Go, eBioscience).

Flow cytometry

The following labelled antibodies were used: anti-CD3, anti-CD4, anti-CD8a, anti-IFN γ , anti-GM-CSF (BioLegend), anti-IL17A, anti-IL17F, anti-IL22, (eBioscience), anti-TNF (BD Bioscience), and fixable viability dye (eBioscience) was used to analyze viable cells. After 6-11 days culture, 0.5×10^6 cells were stimulated with PMA, ionomycin (Sigma-Aldrich), GolgiStop (BD Bioscience) and GolgiPlug (BD Bioscience) for four hours. Fix/Perm kit from BD Bioscience was used for intracellular cytokine staining. Cells were acquired by Fortessa (BD) and analyzed using FlowJo (vX, Tree Star Inc.).

ELISA

Naïve CD4⁺ T cells that were differentiated towards Th17 for 11 days were harvested and after washing, 100,000 cells were restimulated with anti-CD2/anti-CD3/anti-CD28 beads (1:2), in absence of inhibitors. IL-17A secretion was measured in the supernatant after 24 hours. IL-17A secreted by CCR6⁺CD161⁺ enriched Th17 cells was measured in the supernatant after six days of culture. In PBMC and SFMC cultures, cells were harvested after six days of culture without inhibitors and after

washing, 200,000 cells were restimulated in presence of anti-CD2/anti-CD3/anti-CD28 beads (1:2 ratio) and IL-2 (100 IU/mL). IL-17A secretion was measured in the supernatant after 48 hours, using human IL-17A ELISA Ready-SET-Go kit (eBioscience).

RT-PCR

PBMCs from AS patients were cultured for six days to expand Th17 cells. IL-17A⁺ cells were enriched using the IL-17A secretion assay (Miltenyi Biotec), according to manufacturer's protocol. Cells were rested for four days, followed by 48 hours restimulation with anti-CD2/anti-CD3/anti-CD28 beads (1:2 ratio) and IL-2 (100 IU/mL). Prior to PCR amplification a gene specific, 16 cycle preamplification was done using 1-5 ng cDNA and commercially available transcript-specific FAM-labelled probes (Applied Biosystems). Real-time quantitative PCR was performed (Fluidigm Biomark) according to manufacturer's instructions, again using FAM-labelled probes and Taqman Universal PCR Master Mix with UNG. Ubiquitin levels were measured in a separate reaction and used to normalize the data by the Δ Ct method.

Statistics

Statistical differences were determined by Friedman's test, using GraphPad Prism (version 5.01, GraphPad Software), with * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.005$.

Repository Tables

Table E1. IC₅₀ values of MRL248 and MRL-367 in FRET, Gal4 and murine splenocyte assays.

	MRL-248 (IC ₅₀)	MRL-367 (IC ₅₀)
*FRET ROR γ	2 nM	1 nM
Gal4 ROR γ	118 nM	41 nM
murine-splenocyte (IL-17A secretion) (24h / 48h)	13 nM / 10 nM	13 nM / NA

*IC₅₀ were determined using FRET-based assay, Gal4/ROR γ assay and in functional assays tested in Th17 polarization on mouse splenocytes. Methods described in the Repository Methods (“ROR γ -LBD SRC-1 cofactor peptide interaction assay” page 1; “Chimeric ROR γ -GAL4 reporter assay” page 2; “IC50 assay on mouse splenocytes” page 2).

Table E2. Agonist and antagonist activities of MRL-248 and -367 against a panel of nuclear receptors

	MRL-248 (nM)		MRL-367 (nM)	
	Agonist	Antagonist	Agonist	Antagonist
AR	>20000	>20000		
AhR	13500	>20000		
CAR-2	>20000	ND		
CAR-3	>20000	>20000		
ER-alpha	>20000	>20000		
FXR	>20000	>20000		
GR	>20000	>20000		
LXR-alpha	>20000	>20000		
LXR-beta	>20000	>20000		
MR	>20000	>20000		
PR	>20000	>20000		
PXR	>20000	ND		
PPAR-alpha	>20000	>20000		
PPAR-delta	>20000	>20000		
PPAR-gamma	11900	>20000	>10000	>10000
RAR-alpha	4500* (0.1% of ATRA)	>20000		
RAR-beta	1490* partial (5% of ATRA)	5940		
RAR-gamma	1069* partial (10% of ATRA)	3700		
ROR-alpha	>20000	>20000	>5000	>5000
ROR-beta	>20000	>20000		
ROR-gamma	ND	34		
VDR	>20000	>20000		
RXR-alpha	>20000	>20000		
RXR-beta	>20000	>20000		
TR-alpha	>20000	7700	>10000	>10000
TR-beta	>20000	1410	>10000	>10000
ERR-alpha	>20000	>20000		

*RAR-alpha, RAR-beta and RAR-gamma showed saturable responses in agonist mode assays, but extent of activation was ≤10% that of the physiological agonist all-trans retinoic acid (ATRA).

Table E3. Demographics of studied patients.

	Ankylosing Spondylitis (n=35) [†]	Rheumatoid Arthritis (n=16) [§]	Psoriatic Arthritis (n=12) [#]	Inflammatory Bowel Disease (n=16)*	Healthy controls (n=7)
Age, mean (range) years	45.3 (18-65)	57.9 (22-78)	46.8 (32-58)	36 (17-61)	42.4 (30-54)
Sex, male/female	21/14	5/11	4/8	9/7	5/2
HLA-B27+ no. (%)	30 (85.7%)	n/a	n/a	n/a	n/a
anti-TNF therapy, current (previous)	10 (2)	0	2 (2)	6	n/a
BASDAI, mean (range)	4.1 (0.3-10)	n/a	n/a	n/a	n/a
DAS28-CRP, mean (range)	n/a	3.8 (2.1-6.2)	n/a	n/a	n/a
CRP, mean (range)	15.7 (0.1-64.6)	11 (0.4-41.2)	11.3 (0.5-43.4)	n/a	n/a

[†] Blood-derived cells from 29 patients with AS and synovial fluid-derived cells from two additional patients were studied for cytokine production by flow cytometry and/or ELISA; gene transcriptional analysis was performed on an additional four patients. Three AS patients also had IBD, and three had uveitis. [§] 13 patients with RA were studied for cytokine production by flow cytometry and/or ELISA, and gene transcriptional analysis was performed on an additional three patients. [#] Blood-derived cells from 11 patients with PsA were studied for cytokine production by flow cytometry; synovial fluid-derived cells of two of the original 11 and one additional patient were studied for cytokine production by ELISA. * Nine IBD patients had Ulcerative Colitis and seven Crohn's disease.

Legends to Repository Figures

Figure E1. MRL-248 and MRL-37 suppress Th17 polarization in total CD4⁺ T cells from healthy donors.

CD4⁺ T cells isolated to >95% purity from the peripheral blood of healthy controls were cultured under Th17 conditions in presence of MRL-248 or MRL-367 ROR γ t inhibitors. Intracellular IL-17A production was measured at day 6. **(B)** Dose response curve of suppression of Th17 polarization from CD4⁺ T cells. Data are mean + SEM of 6 different donors; *p<0.05, **p<0.01, ***p<0.005 by Friedman's test. **(C)** Relative suppression by MRL-248 or MRL-367 of total IL-17A⁺ cells and IFN- γ ⁺IL-17A⁻ cells. Methods described in the Repository Methods ("Cell culture" page 3; "Flow cytometry" page 4).

Figure E2. ROR γ t inhibitors do not suppress Th17 proliferation from naïve CD4 T cells, but do suppress IL-17A production

FACS sorted (>99% purity) naïve T cells (CD4+CD45RA+CD45RO) or memory T cells (CD4+CD45RA-CD45RO+) from healthy controls were cultured Th17 skewing conditions in presence of ROR γ t inhibitor MRL-248 or MRL-367. IL-17A⁺ cells were determined at day 11 (naïve; **A**) or day 6 (memory; **B**). Naïve T cells from healthy controls were CFSE labelled and cultured for six days in Th17 (*left*) or Th1 (*right*) skewing conditions, in presence of ROR γ t inhibitor MRL-248 or MRL-367. Proliferation was determined at day 6. Data are mean + SEM from 3 different donors. Methods described in the Repository Methods ("Cell culture" page 3; "Flow cytometry" page 4).

Figure E3. MRL-248 and MRL-367 do not alter Th17 phenotype but suppress IL-17A secretion.

CD4 T cells from healthy controls were enriched for Th17 by FACS sorting for CCR6+CD161+ (>98% purity). Cells were cultured for six days with anti-CD2/anti-CD3/anti-CD28 and IL-2 (100 IU/ml) in presence of MRL-248 or MRL-367. IL-17A and IFN- γ as measured by intracellular cytokine staining (**A**

and **B**) and secreted IL-17A by ELISA (**C**). Data are mean + SEM from 2 experiments with 3 different donors. Methods are described in the Repository Methods (“Cell culture” page 3; “Flow cytometry” page 4; “ELISA” page 4).

Figure E4. Th17-promoting culture conditions enhances IL-17A⁺ T cells frequencies of patients with AS.

PBMCs from five AS patients were stimulated for six days with anti-CD2/anti-CD3/anti-CD28 in a 1:20 bead:cell ratio, in presence of IL-2 (100 IU/ml) to promote Th17 expansion. Methods described in the Repository Methods (“Cell culture” page 3; “Flow cytometry” page 4).

Figure E5. ROR γ t inhibition suppresses IL-17A production in healthy control total PBMC cultures.

PBMCs derived from healthy controls (n=7) were cultured for 6 days, and 200,000 cells were subsequently restimulated in presence of ROR γ t inhibitors (MRL-248 or MRL-367; 1 μ M). IL-17A secretion after 48 hours was measured by ELISA. Data are mean + SEM from seven different donors; *p<0.05, **p<0.01 by Friedman’s test. Methods described in the Repository Methods (“Cell culture” page 3; “ELISA” page 4).

Figure E6. ROR γ t inhibition suppresses Th17 polarization of total PBMCs from AS, PsA, RA and IBD.

PBMCs derived from AS, PsA, RA or IBD patients were cultured for six days in presence of ROR γ t inhibitor MRL-248 or MRL-367. Percentage IL-17A⁺ T helper cells of PBMCs from AS patients (n=29), RA (n=13), PsA (n=11) IBD (n=16). Data are mean + SEM; *p<0.01, **p<0.05, ***p<0.005 by Friedman’s test. Methods described in the Repository Methods (“Cell culture” page 3; “Flow cytometry” page 4).

Figure E7. ROR γ t inhibition specifically suppresses IL-17A cells co-producing additional cytokines.

PBMCs derived from AS, RA, PsA or IBD were cultured in presence of RORyt inhibitor MRL-248 or MRL-367 (1 μ M). Intracellular cytokine production in CD4⁺ T cells was measured after six days of culture. Percentage IL-17A⁺ “double positive” and IL-17A⁻ “single positive” cells of IL-17F⁺, GM-CSF⁺, IL-22⁺ or TNF⁺ cells. Data are mean + SEM; *p<0.01, **p<0.05, ***p<0.005 by Friedman’s test. Methods described in the Repository Methods (“Cell culture” page 3; “Flow cytometry” page 4).

Figure E8. IL-17A expression by CD8⁺ T cells is suppressed by RORyt inhibition.

PBMCs derived from AS, RA, PsA or IBD were cultured for six days in presence of RORyt inhibitor MRL-248 or MRL-367 (1 μ M). Intracellular cytokine production in CD8⁺ T cells was measured after four hour stimulation with PMA, ionomycin, brefeldin A and monensin (AS, n=19; RA, n=9; PsA, n=10; IBD, n=16, mean + SEM are shown; *p<0.01, **p<0.05, ***p<0.005 by Friedman’s test.). Methods described in the Repository Methods (“Cell culture” page 3; “Flow cytometry” page 4).

Figure E9. RORyt inhibitor MRL-248 and MRL-367 suppress transcription of Th17 associated genes.

PBMCs from four AS patients (A) and two RA patients (B) were cultured for six days to expand Th17 cells. IL-17A⁺ cells were isolated and rested for four days. Cells were restimulated for 48 hours in presence of MRL-367 or MRL-248 RORyt inhibitor and RT-PCR was performed. Mean + SEM of three technical replicates each from four AS patients and two RA patients are shown. Methods described in the Repository Methods (“Cell culture” page 4; “RT-PCR” page 5).

Figure E10. Schematic cartoon of inhibition culture of tissue-explant derived mononuclear cells.

Mononuclear cells were obtained from a 14 day explant culture of synovial tissue derived from patients with RA and subsequently cultured in presence of anti-CD2/anti-CD3/anti-CD28 beads and RORyt inhibitors. Methods described in the Repository Methods (“Tissue” page 4).

Figure E11. RORyt inhibitors MRL-248 suppress transcription of Th17 associated genes.

Mononuclear cells were obtained from a 14 day explant culture of synovial tissue derived from one RA patients. Cells were restimulated for 48 hours in presence of MRL-248 RORyt inhibitor and RT-

PCR was performed. Three technical replicates of n=1, mean + SD are shown. Methods described in the Repository Methods (“Tissue” page 4; “RT-PCR” page 5).

Figure E1

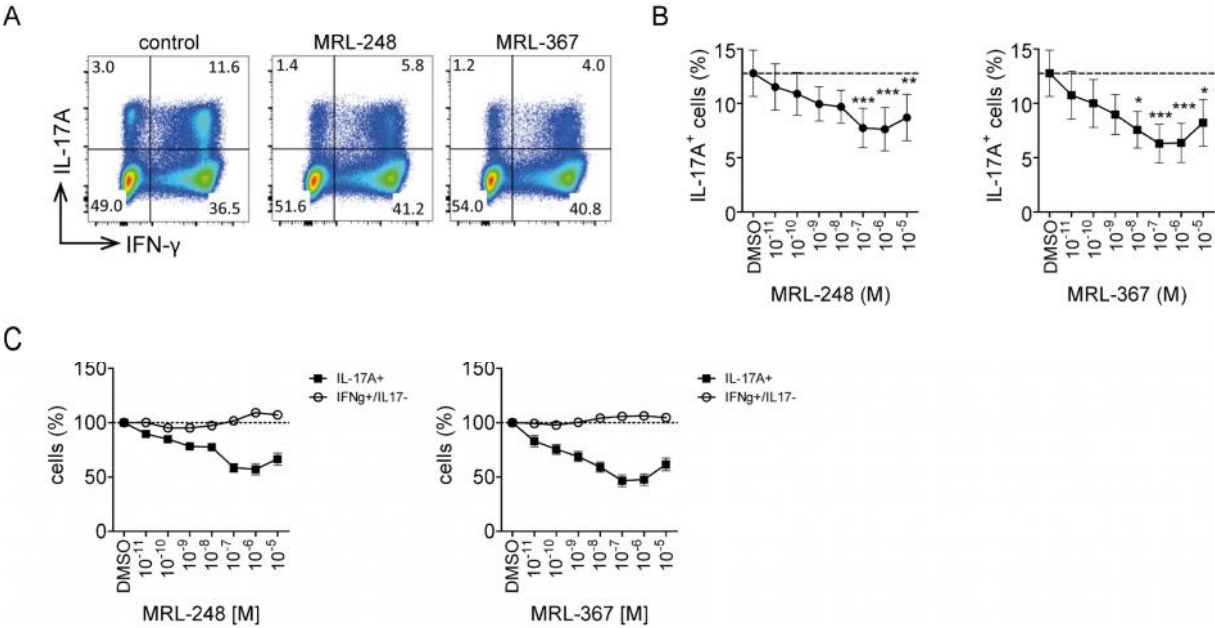


Figure E2

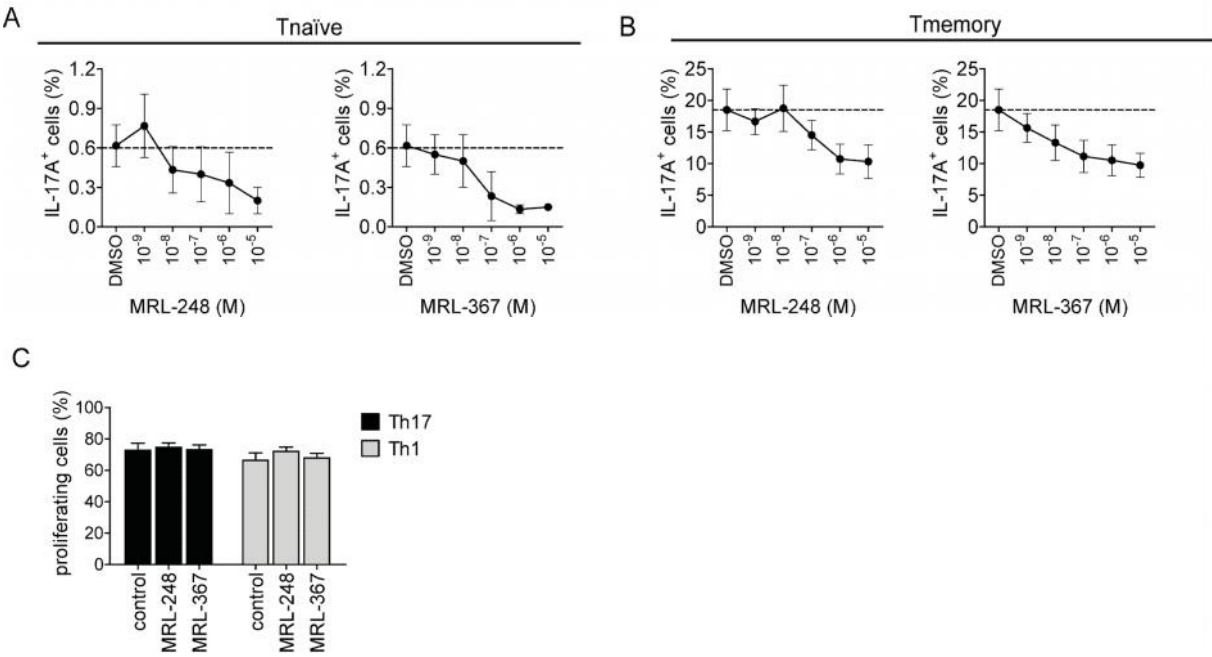
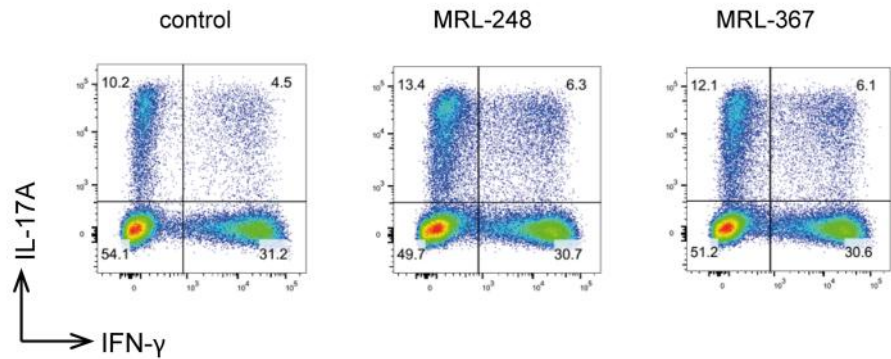
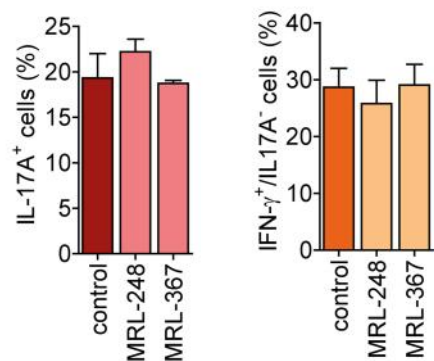


Figure E3

A



B



C

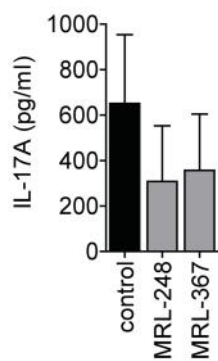


Figure E4

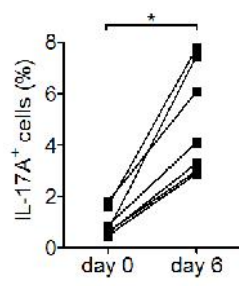


Figure E5

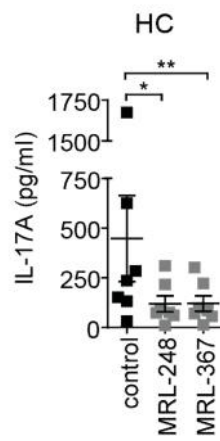


Figure E6

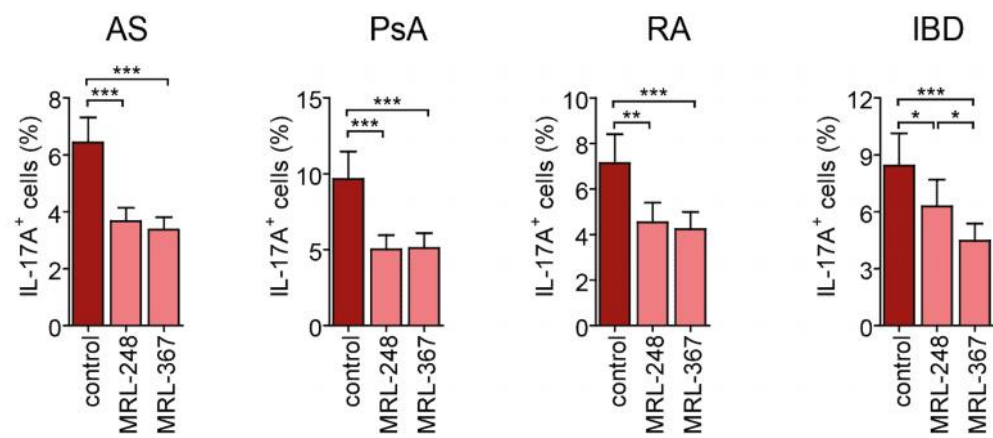


Figure E7

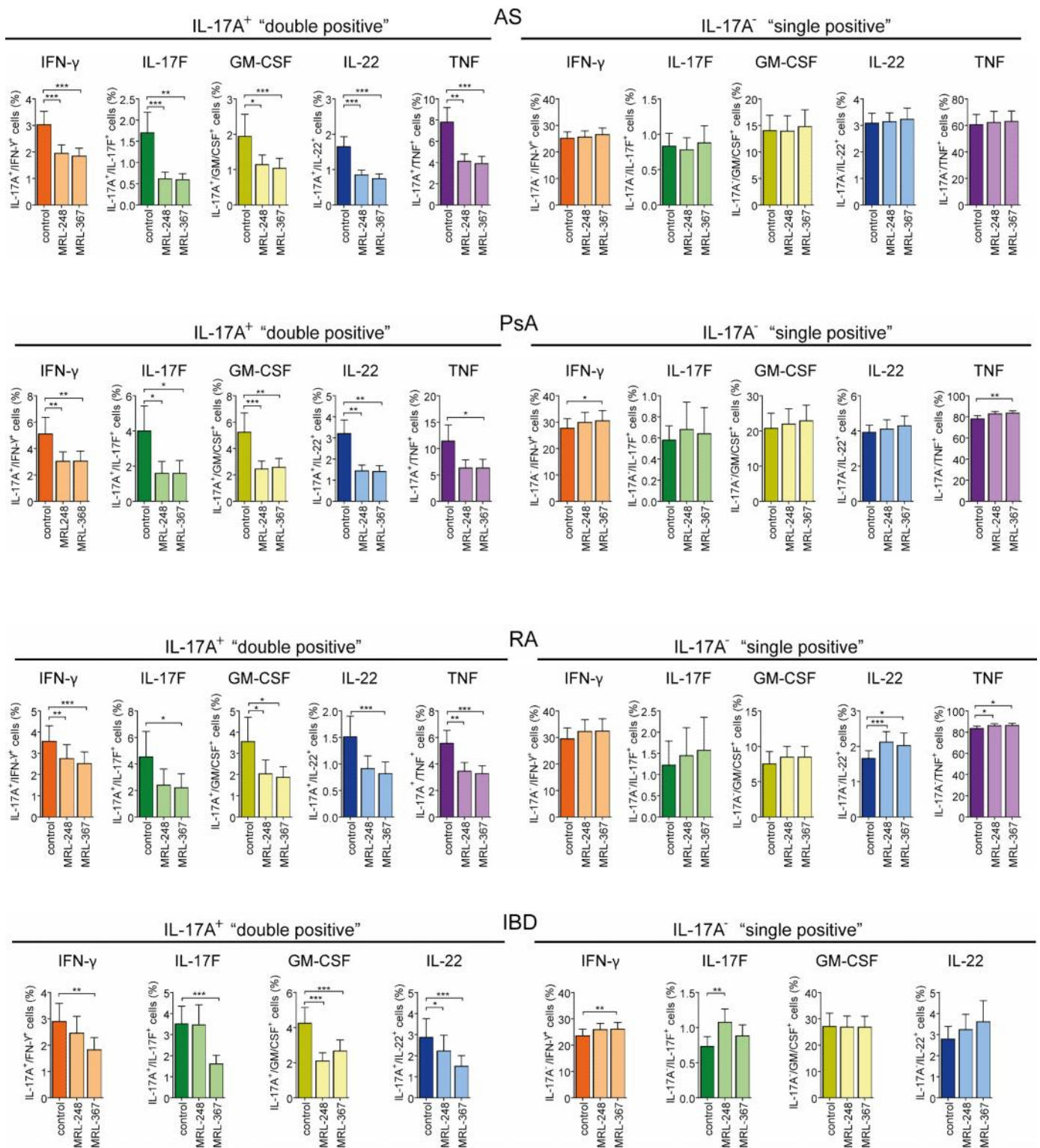


Figure E8

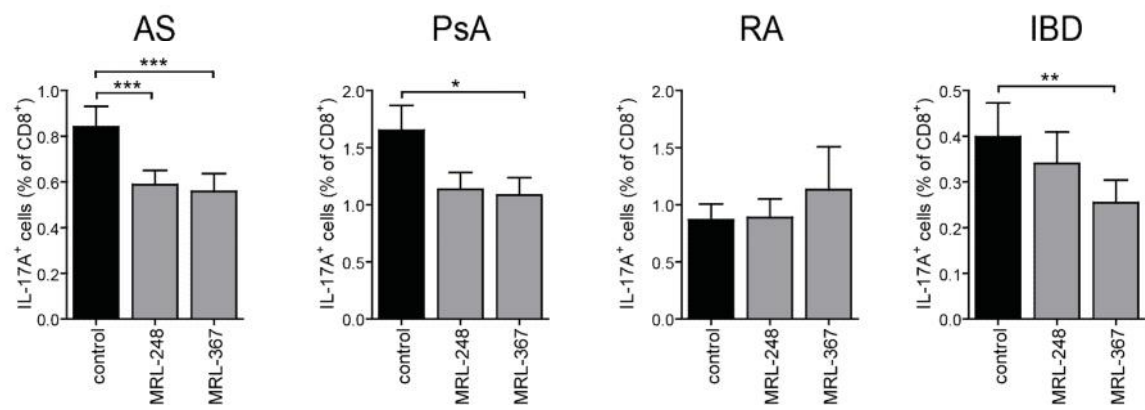


Figure E9

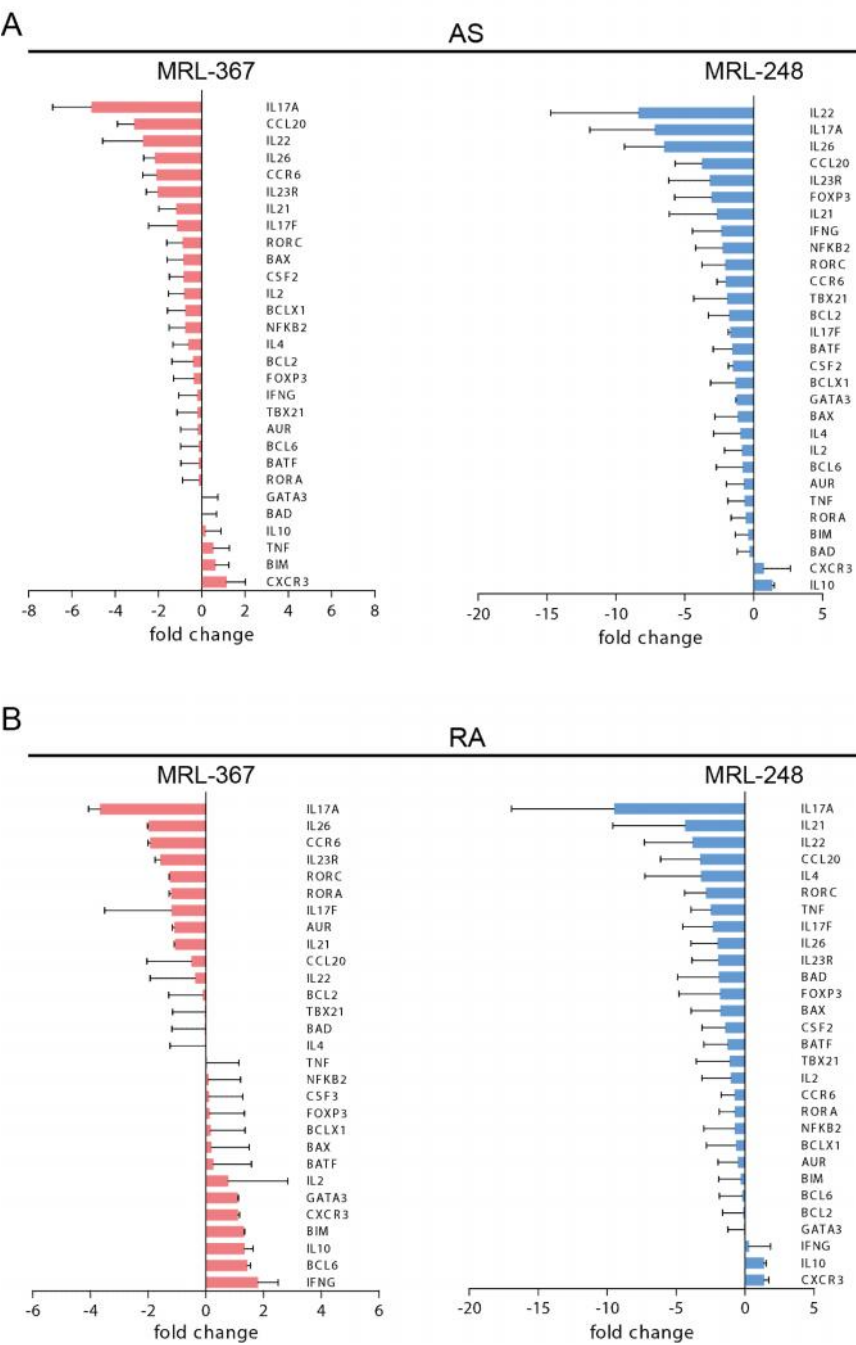


Figure E10

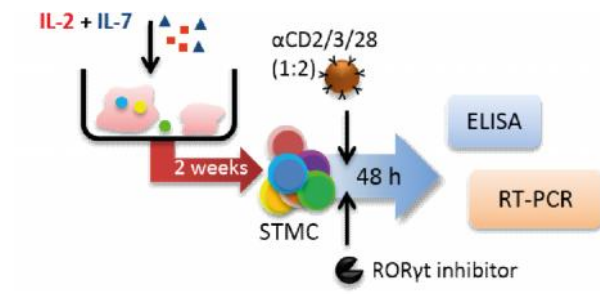


Figure E11

